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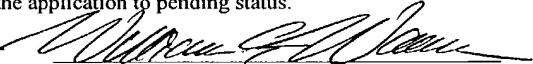
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| Form PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE<br>(Rev 1-98)  |  | Attorney's Docket Number<br><b>18744-0005</b>                          |
| TRANSMITTAL LETTER TO THE UNITED STATES<br>DESIGNATED/ELECTED OFFICE (DO/EO/US)<br>CONCERNING A FILING UNDER 35 U.S.C. 371   |  | U.S. Application No.<br>(if known, see 37 CFR 1.5)<br><b>10/049301</b> |
| International Application No.<br><b>PCT/EP00/07601</b>   | International Filing Date<br><b>August 4, 2000</b> | Priority Date Claimed<br><b>August 6, 1999</b>                         |
| <p>Title of Invention<br/> <b>NON-HUMAN ADENOVIRAL VECTOR FOR GENE TRANSFER, ITS USE AND THE PRODUCTION THEREOF</b> </p>   |  |  |
| <p>Applicant(s) for DO/EO/US<br/> <b>LÖSER, Peter and HOFMANN, Christian</b> </p>  |  |  |
| <p>Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:</p> <ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</li> <li>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</li> <li>3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), and (9) and (21) indicated below.</li> <li>4. <input type="checkbox"/> The US has been elected by the expiration of 19th month from the earliest claimed priority date (Article 31).</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li>a. <input checked="" type="checkbox"/> is attached herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> has been communicated by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ol> </li> <li>6. <input checked="" type="checkbox"/> An English language translation of the International Application into English (35 U.S.C. 371(c)(2))             <ol style="list-style-type: none"> <li>a. <input checked="" type="checkbox"/> is attached herewith.</li> <li>b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</li> <li>c. <input type="checkbox"/> translation not required as the application was filed in English.</li> </ol> </li> <li>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))             <ol style="list-style-type: none"> <li>a. <input checked="" type="checkbox"/> are attached herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been communicated by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input type="checkbox"/> have not been made and will not be made.</li> </ol> </li> <li>8. <input checked="" type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</li> <li>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</li> <li>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</li> </ol> |  |  |
| <p><b>Items 11. to 20. below concern document(s) or information included:</b></p> <ol style="list-style-type: none"> <li>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</li> <li>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</li> <li>13. <input type="checkbox"/> A FIRST preliminary amendment.</li> <li>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</li> <li>15. <input type="checkbox"/> A substitute specification.</li> <li>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</li> <li>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 – 1.825.</li> <li>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</li> <li>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</li> <li>20. <input checked="" type="checkbox"/> Other items or information: Notification of the Recording of a Change</li> </ol>  |  |  |
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PATENT & TRADEMARK OFFICE

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|--|---|--|---|
| U.S. Application No. <b>10704930</b>   | International Application No. <b>PCT/EP00/07601</b> | Attorney's Docket Number <b>18744-0005</b> |   |
| 21. <input checked="" type="checkbox"/> The following fees are submitted:  |   |  |   |
| <b>BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):</b>  |   |  |   |
| Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.. <b>\$1040.00</b>   |   |  |   |
| International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$890.00</b>  |   |  |   |
| International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$740.00</b>   |   |  |   |
| International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$710.00</b>  |   |  |   |
| International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b>  |   |  |   |
| <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b> <b>\$ 890.00</b>   |   |  |   |
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)). <b>\$ 130.00</b>  |   |  |   |
| Claims   | Number Filed  | Number Extra                               | Rate                                    |
| Total claims   | 33 - 20 =   | 13   | x <b>18.00</b> <b>\$ 234.00</b>         |
| Independent Claims   | 2 - 3 =   | 0  | x <b>84.00</b> <b>\$ 0.00</b>           |
| Multiple Dependent Claims (if applicable)  |   | + <b>280.00</b>                            | <b>\$ 280.00</b>                        |
| <b>TOTAL OF ABOVE CALCULATIONS =</b>   |   |  | <b>\$1,534.00</b>                       |
| <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.   |   |  | <b>\$</b>                               |
| <b>SUBTOTAL =</b>  |   |  | <b>\$1,534.00</b>                       |
| Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).   |   |  | <b>\$</b>                               |
| <b>TOTAL NATIONAL FEE =</b>  |   |  | <b>\$1,534.00</b>                       |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$ 0.00</b>   |   |  |   |
| <b>\$40.00 per property</b>  |   |  | <b>+</b>                                |
| <b>TOTAL FEES ENCLOSED =</b>   |   |  | <b>\$1,534.00</b>                       |
|  |   |  | <b>Amount to be refunded:</b> <b>\$</b> |
|  |   |  | <b>charged:</b> <b>\$</b>               |
| <p>a. <input checked="" type="checkbox"/> A check in the amount of <b>\$1,534.00</b> to cover the above fees is enclosed.</p> <p>b. <input type="checkbox"/> Please charge my Deposit Account No. 19-5029 in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 19-5029. A duplicate copy of this sheet is enclosed.</p> |   |  |   |
| NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.  |   |  |   |
| SEND ALL CORRESPONDENCE TO:<br>William L. Warren, Esq.<br>SUTHERLAND ASBILL & BRENNAN, LLP<br>999 Peachtree Street, N.E.<br>Atlanta, Georgia 30309<br>Telephone: 404-853-8000  |   |  |   |
| <br>William L. Warren, Reg. No. 36,714   |   |  |   |
| FORM PTO-1390 (Rev. 1-2002) adapted  |   |  |   |

WO 01/11066

3/P&D

PCT/EP00/07601

NON-HUMAN ADENOVIRAL VECTOR FOR GENE TRANSFER, ITS USE  
AND THE PRODUCTION THEREOF

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Description

The invention relates to the production of a non-human adenoviral vector for gene transfer into mammalian cells. This vector is particularly suitable for the 10 gene transfer into muscle, especially into skeletal muscle, and into cell types which occur in muscle or skeletal muscle. Areas of application are medicine, biotechnology and gene technology. On introduction of functional DNA sequences, the vector is suitable for 15 treating altered, also pathological manifestations in cells or cell complexes, for producing biological material and for vaccination.

Numerous methods and vectors have been developed in the 20 past for gene transfer with the aim of gene therapy or vaccination (review in: Verma, M.I. and Somia, N. (1997), Nature 389, 239-242). Especially favored in this connection for gene transfer, for example with the aim of gene therapy, are vectors derived from 25 retroviruses, adeno-associated viruses (AAV) or human adenoviruses. These types of vectors have a wide range of cell types which can be infected efficiently and are therefore suitable for gene transfer into various tissues.

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The adenoviral vectors of the so-called first generation have been intensively investigated over the last decade as gene transfer vectors (review in: Bramson, J.L. et al. (1995). Curr. Op. Biotech. 6, 590- 35 595). They were derived from the human adenovirus of serotype 5 and have a deletion in the essential E1 region, and often in the nonessential E3 region too, making it possible to insert up to 8 KBp of foreign DNA into the viral genome. These vectors can be produced to

high titers in cells which complement the E1 deficiency, can be easily stored and mediate efficient gene transfer in vitro and in vivo. However, in vivo there is rapid loss of expression of the transgenes and  
5 loss of the vector genome. In addition, extensive tissue toxicity has been observed in some cases after administration of high vector doses. Thus, for example, administration of first generation human adenoviral vectors into murine skeletal muscle leads to leukocyte  
10 infiltration of the infected tissue and to activation of a T-cell subclass (Vilquin et al., Hum. Gene Ther. 6 (1995), 1391-1401; Yang et al., Hum. Mol. Genet. 5 (1996), 1703-1712). Both are caused at least in part by immunological reactions to the viral genes remaining in  
15 the vector. Attempts have therefore been made in particular to take out further early viral genes, but no decisive improvement in in vivo gene transfer was achievable thereby.

20 Recently developed, entirely recombinant human adenoviral vectors (Kochanek, S. et al. (1996). Proc. Natl. Acad. Sci. USA 93; 5731-5736) showed in animal models efficient gene transfer with reduced toxicity and prolonged transgene expression (Morral, N. et al.  
25 (1998), Hum. Gen. Ther. 9: 2709-2716). However, since these vectors also have the normal coat of the human adenovirus and, in the vector genome, the cis elements necessary for replication and packaging - the inverted terminal repeats (IRTs) and the packaging signal -  
30 these vectors still display two limitations which may be regarded as the main problems of human adenoviral vectors. The reasons for both are the human origin of the adenoviruses used and the wide distribution thereof in the human population: (1) the immunity to human adenoviruses which is usually present leads to substantial neutralization and opsonization of the vector. Current studies in animal models have shown  
35 that efficient gene transfer is possible in the presence of this immunity only by use of high vector

doses (Nunes, F.A. et al. (1999), Hum. Gen. Ther. 10, 2515-2526), but this may lead to severe toxic side effects, inter alia including serious hematological side effects (Cichon, G. et al. 1999), J. Gene Med. 1, 5 360-371). (2) In addition, there is the risk of coreplication of the recombinant vector in the event of natural infection with a wild-type adenovirus, with unpredictable consequences. Human adenoviral vectors described at present can therefore be considered for 10 human gene therapy to only a limited extent. One approach to overcoming the problem of immunity to human adenoviruses has recently been found through the development of adenoviral vectors of non-human origin. The viral genome in these vectors is substantially 15 unchanged, and usually relatively small regions nonessential for virus propagation have been replaced by a transgene (WO 97/06826; Mittal, S.K. et al. (1995), J. Gen. Virol. 76, 93-102; Klonjkowski, B. et al. (1997), Hum. Gen. Ther. 8: 2103-2115; Michou, I. et 20 al. (1999), J. Virol. 72, 1399-1410). However, efficient gene transfer with non-human adenoviral vectors in vivo has not as yet been shown experimentally.

25 The problems arising with previously disclosed adenoviral vectors can be solved by using a non-human adenoviral vector which is able to transfer DNA sequences into target cells, especially mammalian cells, and there brings about a preferably transient 30 expression of the transduced gene in the cell or in the organism. The non-human adenoviral vector is particularly suitable for efficient gene transfer in vivo, for example for transduction of mammalian cell types which occur in muscle, or in skeletal muscle. The 35 principal area of use is the transfer of genetic material into cells, for example with the following aims: therapy of genetic and acquired disorders, production of recombinant proteins and vaccination of animals and humans.

One aspect of the invention is thus the use of a non-human adenoviral vector for producing a means for the transfer of genetic material, comprising the coat of a non-human adenovirus and genetic material which is packaged therein and which comprises

- 5 (a) DNA sequences of a non-human adenovirus and
- (b) one or more DNA sequences which code for peptides or polypeptides which are heterologous in relation to the non-human adenovirus, in operative linkage to
- 10 expression control sequences.

The genetic material of the gene transfer vector of the invention comprises DNA sequences from a non-human adenovirus, i.e. of an adenovirus which occurs naturally in a non-human species which is selected, for example, from mammals and birds as listed in Russell, W.C. and Benkő, M. (1999), Adenoviruses (Adenoviridae): Animal viruses. In: Granoff, A. and Webster, R.G. (Eds): Encyclopedia of Virology, Academic Press, London, specifically and in particular adenoviruses from monkeys (SAV, various serotypes), goats (caprine, various serotypes), dogs (CAV, various serotypes), pigs (PAV, various serotypes), cattle (BAV, various serotypes, sheep (OAV1-6 and OAV287) and chickens (FAV, various serotypes) and EDS virus. The virus is preferably an ovine or bovine adenovirus. Examples of suitable ovine adenoviruses are ovine mastadenoviruses or ovine atadenoviruses such as, for example, the OAV isolate 287, whose nucleotide sequence is indicated under Genbank Acc. No. U40389. Suitable bovine adenoviruses are bovine atadenoviruses or bovine mastadenoviruses. Particularly interesting in this connection are bovine and ovine atadenoviruses which are negative in the complement fixation assay.

35 The gene transfer vector of the invention additionally comprises one or more DNA sequences which code for peptide or polypeptides which are heterologous in relation to the non-human adenovirus, in operative

linkage to expression control sequences, i.e. an expression cassette for one or more transgenes. The expression cassette for the transgene can be inserted, for example, into a cloning site.

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The transgene expression cassette preferably comprises the expression control sequences which permit expression in mammalian cells, for example in human cells. The expression control sequences may be 10 constitutively active in the desired target cell or/and be regulable. The expression control sequences may be of viral or cellular origin or comprise a combination of viral and cellular elements. Examples of suitable promoters are viral promoters, for example RSV 15 promoter, CMV immediate early promoter/enhancer, SV40 promoter, or tissue-specific, and in this case especially liver-specific, promoters, for example the human albumin promoter (Ponder et al., Hum. Gene Ther. 2 (1991), 41-52), the human  $\alpha$ -1-antitrypsin promoter/enhancer (Shen et al., DNA 8 (1989), 101-108), the PEPCK promoter (Ponder et al., Hum. Gene Ther. 2 (1991), 41-52) or HBV-derived hybrid promoters, for example EIIImCMV promoter (Löser et al., Biol. Chem. Hoppe-Seyler 377 (1996), 187-193). In addition, the 20 expression-regulating sequences favorably comprise a polyadenylation signal, for example that of the bovine growth hormone gene (Goodwin & Rottman, J. Biol. Chem. 267 (1992); 16330-16334), that of the early transcription unit of SV40 (van den Hoff et al., Nucleic Acids Res. 21 (1993), 4987-4988) or that of the 25 herpes simplex thymidine kinase gene (Schmidt et al., Mol. Cell. Biol. 10, (1990), 4406-4411.

The viral gene transfer vector can be employed for 30 transferring heterologous nucleic acids into permissive cells, cell assemblages, organs and organisms, in particular for gene therapy or for vaccination. For the aim of gene therapy it is possible to use a genomic sequence or the cDNA of a gene whose product is lacking

in the patient to be treated, occurs in nonphysiological quantities or/and is defective. It is also possible to employ a part of a genomic sequence which stretches over a mutation in the target gene and can undergo homologous recombination with the latter.

For the aim of tumor gene therapy it is possible to employ various genes which bring about a slowing of growth or a killing of the tumor cells, where appropriate in combination with drugs or through immunostimulation. For the aim of vaccination it is possible to employ one or more optionally modified genes of a pathogenic organism against which immunization is to be achieved.

The gene transfer vector of the invention may further comprise genetic material from other viruses, for example expressed or regulatory sequences of hepatitis B and hepatitis C virus (HBV, HCV), and from bacteria and pathogenic unicellular or multicellular organisms, for example Plasmodium falciparum.

Preferred specific examples of transgenes are, for the aim of replacement gene therapy, genes for secreted serum factors (for example human coagulation factors IX (FIX) and VIII (FVIII), erythropoietin (Epo)  $\alpha$ -1-antitrypsin (AAT)), and genes for proteins which might be employed for muscle disorders (for example dystrophin, utrophin), and the gene which is defective in Wilson's disease (ATP7B). Preferred specific transgenes for the aim of tumor therapy are tumor suppressor genes p16 or p53 (singly or in combination, for example P16/p53), genes for various interleukins (singly or in combination, for example IL2/IL7) and suicide genes, for example herpes simplex virus type I thymidine kinase (HSV-TK).

The vector is suitable for gene transfer into cells or cell complexes which display altered, also pathological manifestations, for example for gene therapy, for

example for the therapy of congenital or malignant disorders. The vector is likewise suitable for vaccination, for example for vaccination against pathogens, in particular viruses, bacteria and 5 eukaryotic unicellular or multicellular organisms or for vaccination against malignant or nonmalignant cells or cell populations.

It is surprisingly possible to achieve efficient 10 expression of the transgene itself after multiple administration of the vector. This leads to considerable advantages in particular also for applications in the area of gene therapy and vaccination. A further advantage is the observed 15 reduced expression - compared with human adenoviral vectors - of genes of the vector which are intrinsic to the vector after gene transfer into the target cell, there preferably being essentially no expression at all 20 of genes which are intrinsic to the vector after the gene transfer. This leads to a distinct increase in safety for use in the organism, in particular in humans.

An important area of application is the use of the gene 25 transfer vector for obtaining proteins by overexpression in cultivated cells. Because of the very efficient expression of the genes transduced by the vector in cell cultures, for example in IMR-90 and human liver-derived cells, an innovative basis is made 30 possible for the production of recombinant proteins on the industrial scale, which is able to compete with the production in CHO cells which has been chiefly used to date.

35 The vector of the invention can be used to transfer genetic material into a target cell and, preferably, to express this genetic material in the target cell and, preferably, to express this genetic material in the target cell. The target cell is preferably a human

cell. However, it is also possible to use non-human target cells, in particular non-human mammalian cells, for example for applications in veterinary medicine or in research. The gene transfer can take place in vitro,  
5 i.e. in cultivated cells, or else in vivo, i.e. in living organisms or specific tissues or organs of such organisms.

The vector of the invention is particularly preferably  
10 employed for gene transfer into muscle, in particular into skeletal muscle. This finding is all the more surprising since human adenoviral vectors used to date infected skeletal muscle only poorly. The muscle cells are preferably selected from myocytes/myotubes/  
15 myofibers, fibroblasts, dendritic cells, endothelial cells and combinations thereof.

The preferred form of administration of the vector depends on the planned use. For muscle-directed gene  
20 transfer or transfer into a solid tumor, for example, local administration of the vector by intramuscular/intratumoral injection is to be preferred. Systemic introduction is possible for gene transfer into other target organs or tissues, for  
25 example by intraarterial or intravenous injection. Directed transfer into specific tissues or organs can in the latter case take place either by the natural or modified tropism of the vector for particular cell types or by selection of vessels which supply the  
30 tissue to be hit.

The dosage can be decided only after more detailed studies of the efficiency of gene transfer by the particular vector. Typically,  $10^7$  to  $10^{13}$ , for example  
35  $10^9$  to  $10^{11}$ , viral particle/kg of body weight are employed in animal experimental studies. The exact dosage may, however, be modified depending on the nature of the vector, the nature and severity of the disorder and the mode of administration.

In the case where a plurality of dosages of the vector is to be administered at intervals of time, preferably a relatively low dosage of the vector, for example 10<sup>7</sup> 5 to 10<sup>9</sup> viral particles per kilogram of body weight, will be employed for the initial dosages in order to prevent the development of an immune response against the vector, which might make administration of subsequent dosages ineffective. It has surprisingly 10 been found that efficient transgene expression is found even on administration of low vector dosages.

Non-human gene transfer adenoviral vectors can be produced by inserting transgene expression cassettes 15 and, where appropriate, further genetic elements into a basic vector, for example a natural non-human adenovirus or a variant derived therefrom, for example a partially deleted variant and propagation of the resulting vector in suitable permissive cells.

20 The invention is to be explained further by the following figures and examples. These show:

**Figure 1:** Efficient expression of human  $\alpha_1$ -antitrypsin (hAAT) after injection of OAVhaat into the quadriceps muscle. Groups of 5 animals from two mouse strains (Balb/C and C57/Bl-6) received injections of a total of about 1×10<sup>9</sup> infectious particles of OAVhaat in a total volume of 150  $\mu$ l (75  $\mu$ l for each rear leg). Blood was 30 taken three days after the infection, and the concentration of hAAT in the serum was determined by an ELISA. Each bar represents an individual animal.

**Figure 2:** Skeletal muscle is the site of infection by 35 OAV and site of the OAV-mediated gene expression after intramuscular injection of OAVhaat in mice. Balb/C mice were injected with 1×10<sup>9</sup> infectious particles of OAVhaat (2 and 3) or with buffer (1) as described in figure 1, and sacrificed three days after administration. DNA and

RNA were prepared from the injected skeletal muscle and from liver and spleen by standard methods. a) Southern blotting of 20 µg of EcoRI-digested DNA from skeletal muscle, liver and spleen. Hybridization took place with a probe specific for OAV; the position of the EcoRI fragment 2399 bp in size from the OAV vector is indicated. EcoRI-digested OAV DNA, equivalent to 5 copies per cell, was used as positive control. Identical numbers designate identical animals. b) RNase protection assay with 20 µg of total RNA from skeletal muscle. The hybridization took place with a probe which was 364 bases long and spanned the EcoNI fragment of the human aat gene. 20 and 40 pg of an in vitro synthesized haat mRNA were used as positive control; the position of the band specific for the haat transcript is indicated by the arrow.

**Figure 3:** Dose-effect relation of OAV-dependent expression and possibility of intramuscular readministration of OAV vectors in mice. C57/B1-6 mice received the stated amounts of infectious particles of OAVhaat ( $10^9$  to  $3 \times 10^7$ ) in an intramuscular injection. Expression of the haat gene was determined by measuring the concentration of  $\alpha_1$ -antitrypsin in the serum of the mice three days after infection by an ELISA (dark bars). The second injection took place on day 35 after the first administration of the vector; all the animals received a dose of  $5 \times 10^8$  infectious particles. Determination of serum hAAT took place three days after the second dose of vector (pale gray bars).

**Examples:**

1. Material and methods

35    1.1 Cells and viruses

HEK-293 cells (human embryonic kidney cells ATCC CRL-1573), permissive for E1-deleted human adenoviruses) were cultured in DMEM (Gibco BRL) supplemented with 2 mM glutamine and 10% fetal calf

serum at 5% CO<sub>2</sub>. CSL 503 cells (fetal ovine lung cells, permissive for OAV, Pye et al. Austr. Vet. J. 66 (1998), 231-232) were cultured under the same conditions but in 15% fetal calf serum.

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The viral vector used was OAVhAAT which comprises the human  $\alpha_1$ -antitrypsin (hAAT) cDNA under transcriptional control of the Rous sarcoma virus 3'LTR (Hofmann et al., J. Virol 73 (1999), 6930-6936). Ad5haat, an E1-deleted human Ad5 adenoviral vector which comprises the identical haat expression cassette as OAVhaat, was used as control. The viruses were cultured in permissive cell lines and purified as described previously (Sandig et al., Gene Therapy 3 (1996), 1002-1009). Viral titers were examined by an endpoint dilution assay with permissive cell lines. Typical titers for OAV and Ad5 vectors were respectively 0.5 - 1 $\times$ 10<sup>10</sup> and 0.5 - 1 $\times$ 10<sup>11</sup> infectious particles per ml. The ratio of particles to infectious particles for recombinant Ad5 and OAV vectors was 40:1.

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### 1.2 Experimental animals

Eight-week old female Balb/C and C57/Bl-6 mice were purchased from Charles River, Germany. Intramuscular injections were administered into the quadriceps muscle of the mice with a maximum volume of 35  $\mu$ l per injection. The blood was obtained from the external caudal vein in order to determine the serum  $\alpha_1$ -antitrypsin level. The determination of hAAT took place by ELISA as described by Cichon and Strauss (Gene Therapy 5 (1998) 85 - 90).

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The titer of neutralizing antibodies against viral vectors was determined on the basis of the ability of serum to inhibit the infection of 293 cells by human Ad vectors and of CSL503 cells by OAV vectors as described by Hoffmann et al. (1999), supra. The antibody titer

against hAAT in serum was determined by the method of  
Moral et al. (Hum Gene Ther 8 (1997), 1275-1286).

### 1.3 Analysis of DNA and RNA

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DNA and total RNA from mouse tissues and cultivated cells was isolated by using standard methods. Southern blots for detecting OAV-specific DNA in mouse organs was carried out as described previously (Hoffmann et 10 al. (1999), supra) using 20 µg of genomic DNA. RNase protection tests were carried out with 20 µg of total RNA from skeletal muscle using standard procedures. A radiolabeled 362 bp RNA fragment comprising the EcoN1 fragment of human hAAT cDNA was used as probe.

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RT-PCR was carried out with 2 µg of total RNA from skeletal muscle using the Titan™ kit (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions.

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## 2. Results

### 2.1 High expression of serum proteins after injection of a vector derived from OAV into the skeletal muscle of mice

Injection of about  $10^9$  infectious particles of the vector OAVhaat into the quadriceps muscle of Balb/C or C57/Bl-6 mice resulted in concentrations of the 25 transgene (hAAT) in mouse serum which were between 2.6 and 5.9 µg/ml (Balb/C) and 9.8 and 20.0 µg/ml (C57/Bl-6).

In order to demonstrate that the muscle actually is the site both of the infection by the vector and of the expression of the hAAT cDNA, the presence of DNA specific for the vector was carried out by Southern blotting (figure 2a) and of RNA specific for the hAAT transcript was carried out by RNase protection assay

(figure 2b). As is evident from figure 2a, although DNA specific for OAV was detectable in skeletal muscle, it was not in liver and spleen, the organs which would be the primary target of infection on mobilization of the vector into the circulation. It must therefore be assumed that no major amount of the administered vector has reached the system after intramuscular injection. Figure 2b clearly shows that high concentrations of RNA specific for the transgene product hAAT are detectable in skeletal muscle, which identifies the skeletal muscle as the site of hAAT gene expression after intramuscular administration of vector.

The high efficiency of the muscle-specific gene transfer with OAV-derived vectors is evident from figure 3. Although injection of decreasing doses of OAVhAAT into skeletal muscle ( $10^9$  to  $3 \times 10^7$  infectious particles per injection) resulted in decreasing hAAT concentrations in the serum of the corresponding mice, even with the lowest dose ( $3 \times 10^7$  infectious particles) the hAAT concentration reached more than 100 ng/ml. This is a concentration which is higher than the therapeutic concentrations necessary for most therapeutic serum proteins (for example coagulation factors). In addition, with administration of low doses, repeated intramuscular injection of the same vector was possible, which resulted in moderate serum concentrations of hAAT (250-2 300 ng/ml), dark bars in figure 3) with a second administration of  $5 \times 10^8$  infectious particles 35 days after the first administration. The cause of this phenomenon is evidently the induction of only a slight immune response to the vector, in particular on use of a small dose of vector.

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2.2 *Induction of a humoral immune response to the transgene product after injection of an OAV-derived vector into the skeletal muscle of mice*

Efficient expression of the transgene and presentation of the antigens encoded by the transgene is necessary for use of a vector for vaccination. The efficiency of vaccination is reflected by the titer of antibodies 5 against the transgene/antigen. The potential for OAV as a vaccination vector was determined by a simple experiment.

Balb/C mice received an intramuscular injection of  $5 \times 10^8$  10 infectious particles of OAVhAAT. On day 30 after the infection, the titers of antibodies against the transgene product, human  $\alpha_1$ -antitrypsin, in the serum of the mice were determined as follows: 96-well plates were coated with 100  $\mu$ l of an antibody directed against 15 hAAT (DiaSorin, Stillwater, USA, Cat. No. 80852) at 37°C for 1 h, and incubated with blocking buffer (5% skim milk powder in TBS, 0.05% Tween 20) at 4°C overnight and then with an hAAT standard (100 ng/ml in 100  $\mu$ l) at 37°C for 2 h. Dilutions of the appropriate 20 mouse sera (1:10 to 1:100 000, in a volume of 100  $\mu$ l) were then put onto the plate, and the plates were incubated at 37°C for 2 h. 100  $\mu$ l of a peroxidase-coupled rabbit antibody directed against mouse IgG (Pierce, Rockford, USA) were then added, and the 25 incubation was continued for 2 h. The enzymic activity was determined by standard methods using OPD as substrate; the absorption was measured at 595 nm. The titer of antibodies against hAAT was greater than 100 000 in the serum of all the investigated mice of 30 the Balb/C strain ( $n=5$ ) after an injection of about  $10^9$  infectious particles. The antibody titer in the serum from mice of the C57/Bl-6 strain ( $n=5$ ) was between 100 000 and 10, corresponding to the dose of the vector OAVhaat administered (see table 1). Induction of 35 antibodies directed against hAAT in the latter mouse strain is remarkable because this strain produced no antibodies against the transgene product after infection with human adenoviral vectors which transduce

the hAAT gene (Michou et al., Gene Therapy 4 (1997), 473-482; Morral et al., (1997) *supra*).

Table 1. Titers of antibody against hAAT in the serum  
5 of mice (C57/Bl-6) which received an intramuscular  
injection of the stated dose of OAV.

| Dose administered | Titer of antibodies against hAAT |
|-------------------|----------------------------------|
| $1 \times 10^9$   | 1:10 000                         |
| $5 \times 10^8$   | 1:10 000 - 1:1 000               |
| $2.5 \times 10^8$ | 1:1 000                          |
| $1.2 \times 10^8$ | 1:100-1:10                       |
| $6 \times 10^7$   | 1:100                            |
| $3 \times 10^7$   | 1:100                            |

2.3 No detectable expression of OAV genes after  
10 infection.

In order to detect whether injection of OAV in skeletal muscle of mice leads to expression of early to late OAV genes, total RNA was obtained from skeletal muscle 20 and 40 h after infection and was analyzed for OAV-specific transcripts by RT-PCR. An infection with the human adenovirus Ad5haat ( $10^{10}$  infectious particles) and a corresponding detection of transcripts was carried out in control animals.

Neither early OAV genes (E4 gene, DNA binding protein gene) nor late OAV genes (hexon gene and pVII gene) were detectable in mouse skeletal muscle cells infected with OAVhaat. In contrast to this it was possible to detect in skeletal muscle cells infected with Ad5haat PCR products of the E4orf6 gene (20 and 40 h after infection) and of the DNA binding protein gene (20 h after infection).

Thus, whereas viral genes of the Ad5 vector are expressed in mouse skeletal muscle, no expression of viral genes of OAV was detectable.

2.4 Efficient expression of genes transduced by means  
of a non-human adenoviral vector in the cell  
culture system

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IMR 90 cells (Nichols et al., Science 196 (1977), 60-  
63) were infected with OAVhaat at an MOI of 1. After  
3 days, expression of recombinant hAAT was determined  
quantitatively by an ELISA. The determined amount of  
10 the recombinant protein was 1 mg/ml of cell culture  
supernatant/ $10^6$  cells.

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Dec. 3, 2001

**Patent claims**

1. The use of a non-human adenoviral vector for producing a means for the transfer of genetic material by local injection into muscle cells or muscle cell complexes of mammals which display altered, also pathological manifestations, or into tumors, for the therapy of congenital, acquired or malignant disorders, comprising the coat of a non-human adenovirus and genetic material which is packaged therein and which comprises
  - (a) DNA sequences of a non-human adenovirus and
  - (b) one or more DNA sequences which code for peptides or polypeptides which are heterologous in relation to the non-human adenovirus, in operative linkage to expression control sequences.
2. The use as claimed in claim 1,  
**characterized in that**  
the virus is an adenovirus from a non-human species selected from mammals and birds.
3. The use as claimed in claim 2,  
**characterized in that**  
the virus is an ovine or bovine adenovirus.
4. The use as claimed in claim 3,  
**characterized in that**  
the virus is an ovine or bovine mastadenovirus or atadenovirus.
5. The use as claimed in claim 3 and 4,  
**characterized in that**  
the ovine adenovirus is the OAV isolate 287.
6. The use as claimed in any of claims 1 to 5 for transferring genetic material into human cells.

*Amended sheet*

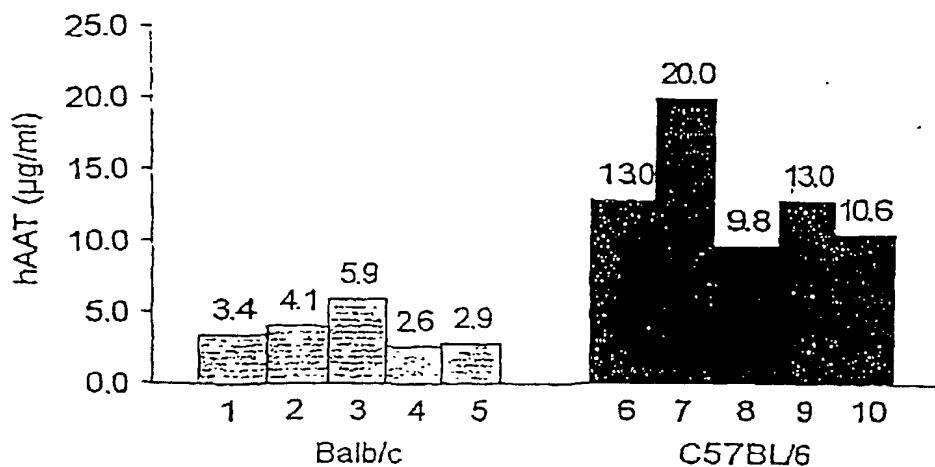
7. The use as claimed in any of claims 1 to 6 for transferring genetic material into skeletal muscle.
8. The use as claimed in any of claims 1 to 7, characterized in that the muscle cells are selected from myocytes/myotubes/myofibers, fibroblasts, dendritic cells, endothelial cells and combinations thereof.
9. The use as claimed in any of claims 1 to 8, characterized in that the vector is administered more than once.
10. The use of a non-human adenoviral vector for producing a means for the transfer of genetic material for the production and isolation of recombinant proteins in cell culture, comprising the coat of a non-human adenovirus and genetic material which is packaged therein and which comprises
  - (a) DNA sequences of a non-human adenovirus and
  - (b) one or more DNA sequences which code for peptides or polypeptides which are heterologous in relation to the non-human adenovirus, in operative linkage to expression control sequences.

**Abstract**

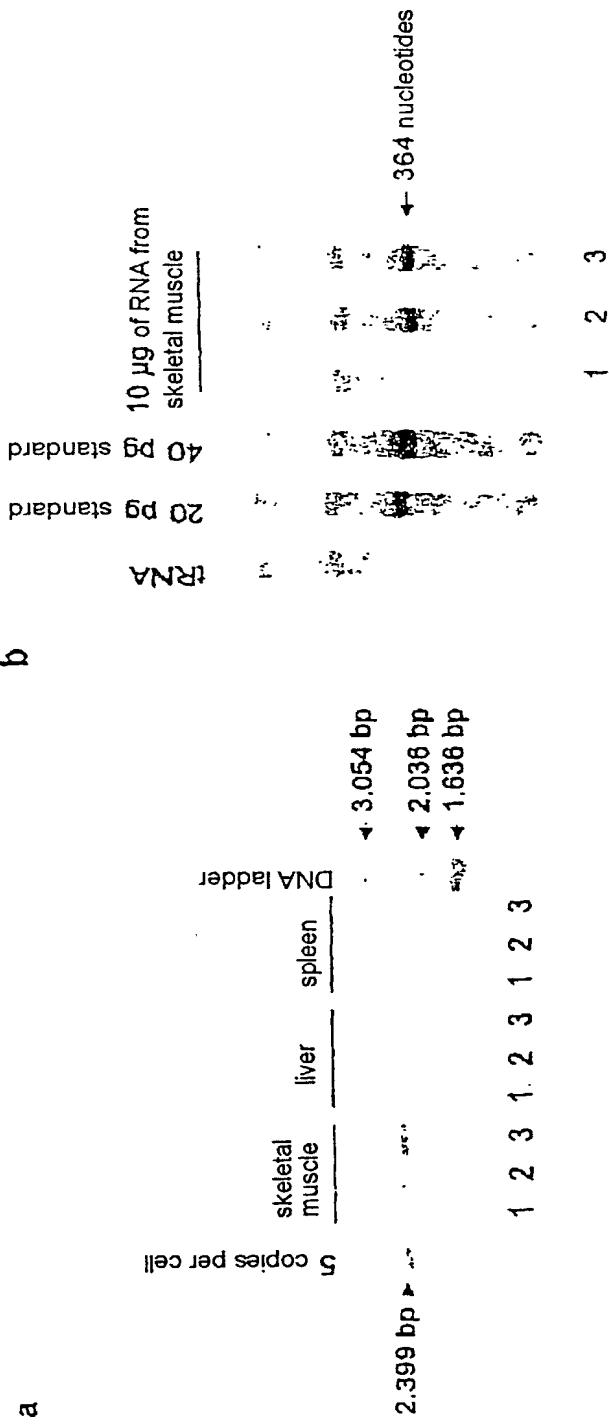
The invention relates to the production of a non-human adenoviral vector for gene transfer into mammalian cells. This vector is particularly suitable for gene transfer into muscle, in particular into skeletal muscle, and into cell types which occur in muscle or skeletal muscle. Areas of application are medicine, biotechnology and gene technology. On introduction of functional DNA sequences, the vector is suitable for treating altered, also pathological manifestations in cells or cell complexes, for producing biological materials and for vaccination.

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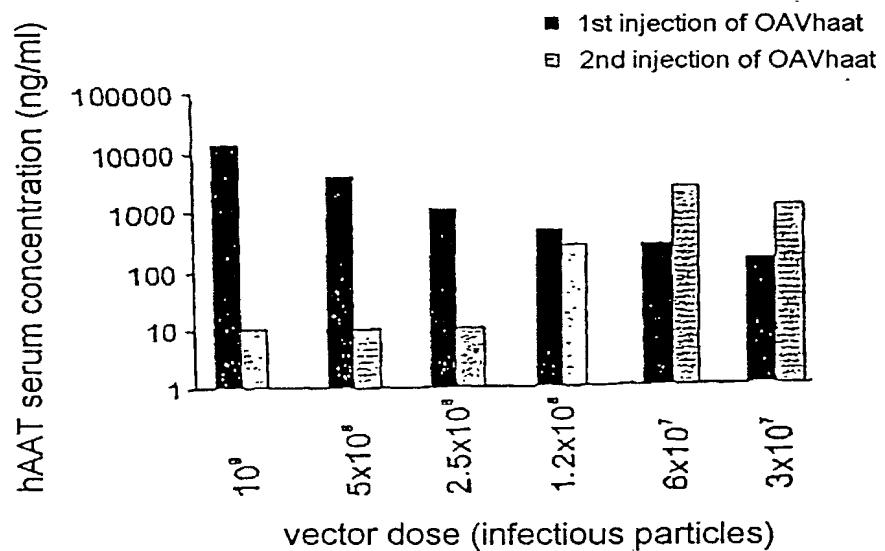
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(Foreign associate use only)

**DECLARATION AND POWER OF ATTORNEY**

Attorney's Docket No. \_\_\_\_\_

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **Non-human adenoviral vector for gene transfer, its use and the production thereof**, the specification of which

is attached hereto.

was filed on **August 04, 2000** as U.S. Application or PCT International Application No. **PCT/EP 00/07601** and was amended (if applicable) on **December 03, 2001**.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I do not know and do not believe that the same was ever known or used by others in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to the date of this application. I further state that the invention was not in public use or on sale in the United States of America more than one year prior to the date of this application. *I understand that I have a duty of candor and good faith toward the Patent and Trademark Office*, and I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate disclosing subject matter in common with the above-identified specification and having a filing date before that of the application on which priority is claimed:

| Country | App. No.     | Date of Filing  | Priority Claimed Under 35 USC §119 |
|---------|--------------|-----------------|------------------------------------|
| DE      | 199 38 332.4 | August 06, 1999 | X Yes No _____                     |
| DE      | 100 19 006.5 | April 17, 2000  | X Yes No _____                     |

I hereby claim the benefit under Title 35, United States Code, § 120 of any prior United States application(s), or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each claim of the present application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56, which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application No. \_\_\_\_\_ Filing Date \_\_\_\_\_ Status: patented, pending, abandoned

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statement were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patents issuing thereon.

I hereby authorize the U.S. attorneys named herein to accept and follow instructions from Holland & Holland as to any action to be taken in the Patent and Trademark Office regarding this application, without direct communication between the U.S. attorney and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney named herein will be notified by the undersigned.

POWER OF ATTORNEY: The following attorneys are hereby appointed to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Peter G. Pappas - 33,205; Daniel J. Warren - 34,272; William L. Warren - 36,714; Michael S. Pavento - 42,985; Lisa M. Coborn - 44,669; Robert A. Lester - 24,004; Erinn C. Kelly - 44,822; Jerry C. Liu - P47,754; Kevin W. King - 42,737.

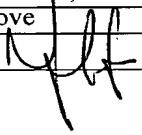
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| Date: <u>X 28.01.02</u>  |

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Title: \_\_\_\_\_  
Page 2

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